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Phenylacetic and Phenylpropionic Acids Do Not Affect Xylan Degradation by *Ruminococcus albus*

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Since the addition of either ruminal fluid or a combination of phenylacetic and phenylpropionic acids (PAA/PPA) has previously been shown to dramatically improve cellulose degradation and growth of *Ruminococcus albus*, it was of interest to determine the effects of these additives on xylan-grown cultures. Although cell-bound xylanase activity increased when either PAA/PPA or ruminal fluid was added to the growth medium, total xylanase did not change, and neither of these supplements affected the growth or xylan-degrading capacity of *R. albus* 8. Similarly, neither PAA/PPA nor ruminal fluid affected xylan degradation by multiple strains of *R. albus* when xylan prepared from oat spelts was used as a carbohydrate source. These results show that the xylanolytic potential of *R. albus* is not conditional on the availability of PAA/PPA or other components of ruminal fluid.

Ruminococcus albus is a gram-positive anaerobe widely recognized for its high cellulolytic activity. A distinguishing feature of *R. albus* isolates is their dependence on the provision of micromolar concentrations of phenylacetic and phenylpropionic acids (PAA/PPA) for optimal rates of growth and cellulose degradation (12, 16, 18, 19, 20). PAA/PPA appear to be necessary for the formation of cell-associated, high-molecular-weight protein complexes believed to be cellulosomes (13). Many isolates of *R. albus* have also been shown to degrade xylan and the hemicellulose fraction of plant cell walls (3, 9). Greve et al. (11) demonstrated that *R. albus* strain 8 produces several enzymes involved in xylan degradation, including β-1,4-xylanase, β-xylosidase, and α-arabinofuranosidase. The strain was also shown to ferment glucose and xylose residues present in alfalfa cell wall preparations in preference to other sugars (11). However, there are no data on the possible effect(s) from either PAA/PPA or other components of ruminal fluid on xylan degradation and growth of *R. albus.* Considering that heteroxylans represent a major part of the plant cell wall, it was of interest to determine whether optimal rates of *R. albus* growth, as well as xylan degradation, would be conditional on the provision of PAA/PPA or ruminal fluid.

Bacterial strains and growth experiments. *R. albus* strains 8, B199, and 7 were obtained from the culture collection at the National Center for Agricultural Utilization Research, U.S. Department of Agriculture, Peoria, Ill. In the experiments described here the strains were cultured at 39°C in a semidefined medium, described by Champion et al. (5), containing 5% (vol/vol) clarified ruminal fluid (RF) or the same medium with ruminal fluid omitted but supplemented with either 25 μM each of PAA and PPA (PA) or sterile water (WO). Carbohydrate sources were included at a concentration of 0.4% (wt/vol). Pebble-milled Whatman No. 1 filter paper was used in cellulose-containing media, and the xylan preparations (birchwood and oat spelt) were purchased from Sigma Chemical Co., St. Louis, Mo. The bacterial strains were passed no less than three times in the respective medium before each experiment.

In experiments with *R. albus* strain 8, the WO, PA, and RF media were prepared in duplicate 500-ml anaerobic bottles (Bellco Glass, Vineland, N.J.) fitted with a serum bottle closure that can be sealed with a butyl rubber stopper and aluminum seal. At each sampling time the bottles were mixed and a 10-ml sample was collected anaerobically by using aseptic procedures. Disposable sterile pipettes, with their tips broken off to ensure no impediment to the collection of the cellulose or xylan, were used to collect samples. Residual cellulose was measured by the anthrone procedure (10). Water-soluble and -insoluble forms of residual xylan were precipitated by the addition of 1 M perchloric acid to culture samples, and after centrifugation they were measured by the orcinol procedure (10). Bacterial growth was determined by recovering bacterial cells by centrifugation, washing the pellets twice with 1% (wt/vol) KCl, and, after boiling in 1% (wt/vol) 3-[(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonate solution for 20 min, measuring total protein by the method of Bradford (2), with bovine serum albumin used as a standard.

As expected, the rate of cellulose degradation by *R. albus* 8 was dramatically improved in PA and RF cultures, the cellulose solubilization rates being 0.86, 3.05, and 3.11 mg/ml/h for WO, PA, and RF cultures, respectively. Bacterial growth was also improved in PA and RF cultures (data not shown) in a manner similar to that of previous findings (12). The results verified that *R. albus* 8 still requires PAA/PPA for maximal rates of cellulose degradation and growth. In the first experiment with birchwood xylan-containing cultures, samples were collected every 6 h for a total of 72 h. Much of the cell growth and xylan degradation occurred within the first 30 h of incubation (data not shown), so a second experiment was con-

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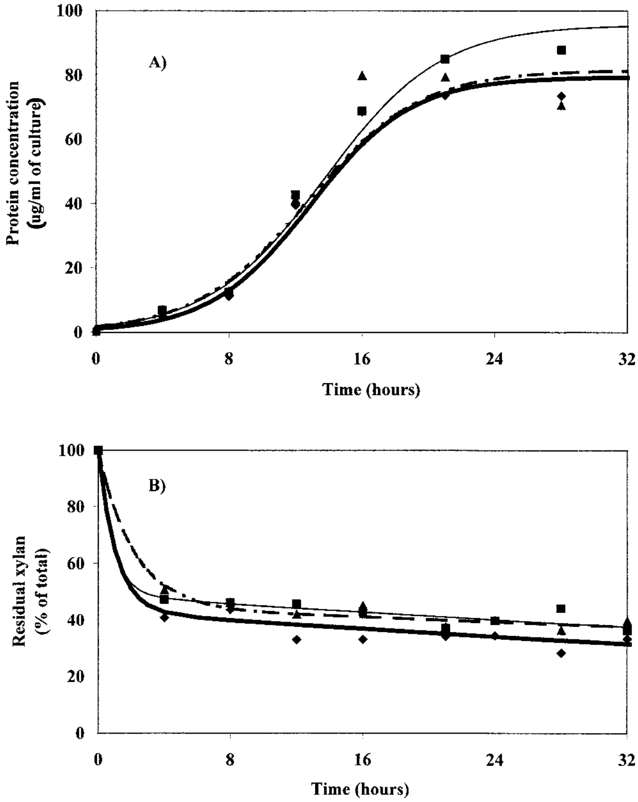

FIG. 1. Time course measurement of *R. albus* 8 growth (A) and residual xylan (B) in a semidefined medium (5) prepared with either WO (▲), PA (■), or 5% (vol/vol) RF (♦). Data points represent the averages of the values obtained for samples from two cultures and analyzed in triplicate ($n = 6$). The lines represent the growth patterns predicted for WO (dashed line), PA (thin solid line), and RF (thick solid line) from using the values fitted to a logistic model (21).

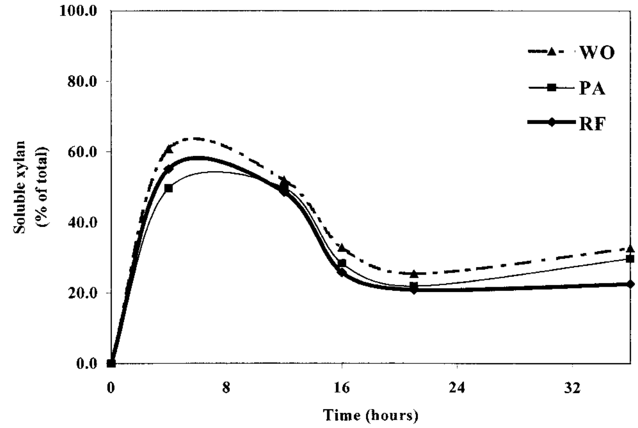

FIG. 2. Time course measurement of total xylooligosaccharides present in culture fluids following growth of *R. albus* 8 in WO (▲), PA (■), or RF (♦) medium. Data points represent the average values obtained from samples of two cultures analyzed in duplicate ($n = 4$).

ducted, with sampling intervals decreased to every 4 h for a total of 36 h. Xylan degradation and bacterial growth from these experiments are illustrated in Fig. 1. The degradation of acid-insoluble xylan was rapid and largely complete within the first 4 h of incubation, and notably, there was little difference in the rate or extent of xylan degradation among the WO, PA, and RF cultures, nor were there differences in bacterial growth.

In all three media the concentration of acid-soluble sugars increased rapidly, reaching maximal levels within 4 h (Fig. 2) but declining over the next 16 h as bacterial growth proceeded. After 20 h of incubation, however, bacterial growth ceased and the concentrations of the acid-soluble and -insoluble forms of carbohydrate remained largely unchanged for the remainder of the incubation period. The xylooligosaccharide profile in the three types of cultures was analyzed by thin-layer chromatography following previously described methods (7). The oligosaccharides were developed (one ascent) in a solvent of 6:1:1:2 (vol/vol) 2-propanol, ethyl acetate, nitromethane, and water, which effectively resolved xylose (X_1) through xyloheptaose (X_7), and these were visualized with an orcinol spray reagent (10 ml of H_2SO_4, 90 ml of methanol, 0.2 g of orcinol) followed by heating to 100°C (7). The profiles are shown in Fig. 3. There were no measurable xylooligosaccharides present in the samples prior to inoculation (time zero). The samples collected 8 h postinoculation contained xylose (X_1) to xylohexaose (X_6), and there were no differences in the profile among the three types of cultures. After 21 h of growth, only xylose and a trace amount of xylobiose were evident, and neither arabinose nor glucose, which are not present in birchwood xylan, was detected.

Collectively, these data support the contention that there is no influence of PAA/PPA (or other components present in ruminal fluid) on xylan degradation, the profile of soluble xylooligomers produced, or growth of *R. albus* 8. Xylan degradation was also incomplete, suggesting that the carbohydrate composition of the residual xylan may be recalcitrant to further hydrolysis and that growth by *R. albus* 8 is terminated as a result. To further address the reason(s) underpinning incomplete xylan degradation, *R. albus* 8 was cultured in WO, PA, and RF media for 24 h, and then 2-ml samples of each culture were taken for measurement of residual xylan. The remainder (8 ml) of each culture was then centrifuged (2,500 × *g* for 20 min), and the supernatant fraction was carefully removed with a sterile, stainless steel needle inserted through the butyl rubber closure of each tube. The pelleted bacterial cells and residual xylan were resuspended in 10 ml of sterile, anaerobically prepared WO, PA, and RF media that did not contain xylan. The cultures were reincubated for another 24 h, and then the residual xylan was determined as described above. After 24 h of incubation, xylan degradation was 44, 49, and 45% in WO, PA, and RF cultures, respectively. After the addition of fresh medium, 85, 93, and 87% of the xylan was degraded in WO, PA, and RF media, respectively, showing that the cessation of xylan degradation and growth is not attributable to alterations in xylan composition but is perhaps due to xylose accumulation. Furthermore, neither PAA/PPA nor other components of ruminal fluid result in physiological changes that result in enhanced xylan degradation or bacterial growth.

Enzyme assays. Measurements of xylanase and xylosidase activities produced by *R. albus* 8 are presented in Table 1. Xylanase activity was measured with birchwood xylan as the substrate, which was prepared as a 1% (wt/vol) suspension in 0.1 M $NaPO_4$ buffer (pH 7). All assays were conducted aero-

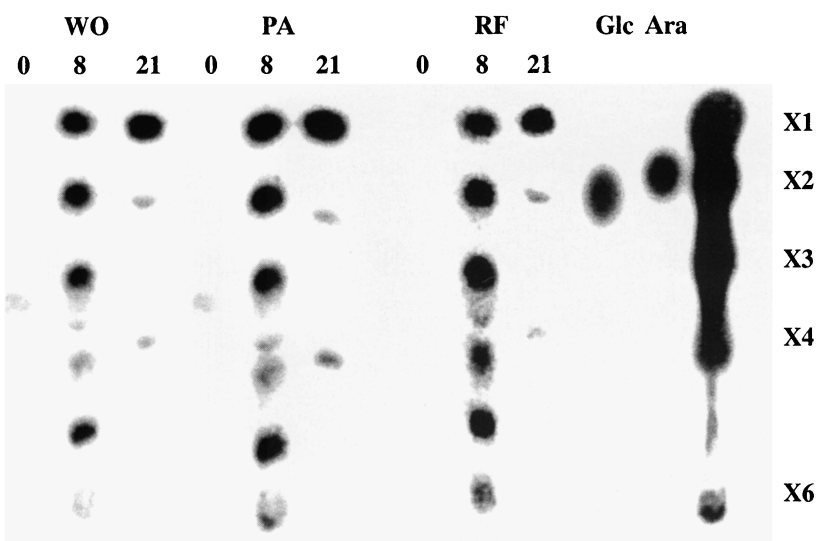

FIG. 3. Thin-layer chromatography analysis of the soluble xylan degradation products produced during growth of *R. albus* 8 in medium prepared to contain either RF, 25 μM PA, or WO. The samples analyzed were collected at 0, 8, and 21 h after inoculation. Mixtures of xylose (X_1) and xylooligomers (X_2 to X_6) and of arabinose (Ara) and glucose (Glc) were used as standards. The oligosaccharides contained in the samples were identified by comparison to the thin-layer chromatography of authentic standards (Megazyme, Wicklow, Ireland).

bically at 39°C, and the linear range of these assays with respect to protein concentration and time was first determined. The reducing sugars released in 15 min were measured by using the dinitrosalicylic acid procedure (15), and xylose was used to produce a standard curve. One unit of enzyme activity was defined as 1 μmol of reducing sugar released per ml of culture. The amount of total xylanase activity produced by *R. albus* 8 was similar in all three cultures, although more activity remained cell associated when bacteria were cultured in PA and RF media than in WO medium (Table 1). PAA/PPA and ruminal fluid appeared to affect enzyme retention rather than enzyme production, but these changes did not result in improved xylan degradation or bacterial growth (Fig. 1). Xylosidase activity was determined by measuring the release of *para*-nitrophenol (pNP) from pNP-β-D-xylopyranoside (pNPX; obtained from Sigma). Total cellular proteins (45 to 60 μg) from cultures harvested in the late logarithmic phase of growth were added to 200 μl of substrate (2.5 mM pNPX in 50 mM $NaPO_4$ buffer [pH 6.8]) and were incubated at 39°C for 30 min. One unit of enzyme activity was defined as 1 nmol of pNP released per ml of culture. Xylosidase activity was relatively low in all samples, and it appears that neither PAA/PPA nor ruminal fluid stimulated the production or retention of this enzyme on the bacterial cell surface.

TABLE 1. Xylosidase and xylanase activities of *R. albus* strain 8 following growth in xylan-containing cultures containing either no additions (WO), PA, or 5% (vol/vol) RF

Enzyme	Activity in medium:		
	WO	PA	RF
Xylosidase[a]			
Cell associated	36 ± 10.0	20 ± 5.0	15.6 ± 5.0
Extracellular broth	1.9 ± 0.3	3.7 ± 0.2	2.8 ± 0.3
Xylanase[b]			
Cell associated[c]	0.8 ± 0.1	2.3 ± 0.3	2.5 ± 0.3
Extracellular broth	3.3 ± 0.2	2.0 ± 0.3	2.0 ± 0.3

[a] One unit of activity is defined as 1 nmol of *p*-nitrophenol released per ml of culture.
[b] One unit of activity is defined as 1 μmol of reducing sugar released per ml of culture.
[c] Main effect of RF = PA > WO.

Concluding remarks. These experiments show for the first time that neither PAA/PPA nor other unidentified compound(s) in ruminal fluid are needed to maximize xylan degradation by *R. albus* 8 or its growth on xylan degradation products. While PAA/PPA and ruminal fluid did not greatly affect total xylanase production by *R. albus* 8, it did result in a change in the location of xylanase activity, with more remaining cell associated. The cellulosome from *R. albus* F-40 has been shown to contain xylanases (17), but the xylanases cloned from *R. albus* isolates do not possess, to date, dockerin domains, suggesting there are also noncellulosomal forms. Further studies may help identify whether the multiple xylanases present in *R. albus* are subject to different types of regulatory control. However, when washed cell suspensions from WO, PA, and RF cultures were used to inoculate xylan-containing medium, there were still no differences in xylan degradation, suggesting that xylanase per se is not rate limiting to *R. albus* growth.

Birchwood xylan was specifically chosen for these experiments because it is prepared by extraction with ethanol and base, should not be acetylated, and by mass is greater than 90% linear xylose polymers with little or no arabinose present (7). As such, it was presumed to be the most homogeneous form of xylan available to examine the xylanolytic potential of *R. albus*. However, the noncellulosic polysaccharides of most plant species consumed by ruminants and herbivores are much more heterogeneous in composition, and it seemed possible that the stimulatory effects of PAA/PPA or ruminal fluid might be

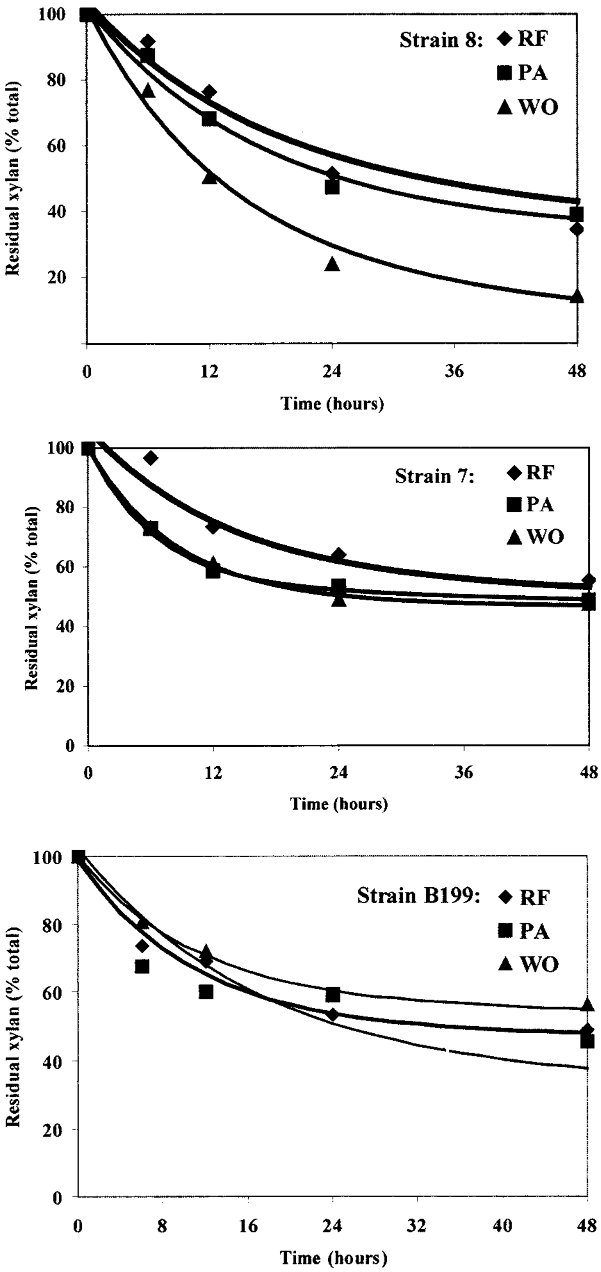

FIG. 4. Time course measurement of the degradation of oat spelt xylan by *R. albus* strains 8, 7, and B199 during growth in RF, PA, and WO media. Samples were taken from duplicate cultures at the times shown, and residual xylan was quantified by using the orcinol-based colorimetric assay as described in the text (7).

obscured due to the xylan source used in these experiments. To address this concern, three different *R. albus* strains were cultured in the WO, PA, and RF media prepared with oat spelt xylan, and xylan degradation was measured from samples taken over 48 h, as described above. The results of these studies are shown in Fig. 4, and neither PAA/PPA nor other compounds present in ruminal fluid enhanced the xylan-degrading capacity of any of the *R. albus* strains examined. Furthermore, the degradation patterns observed here are similar to those seen by Dehority with *R. albus* 7 by using hemicellulose preparations (8, 9). Based on these findings, we conclude that the findings made with *R. albus* 8 are typical of other *R. albus* isolates with respect to polysaccharide degradation and that the findings with birchwood xylan are not confounded by either the source or composition of this substrate.

Although *R. albus* 8 produces both xylose and xylooligosaccharides, it only utilizes the latter as a carbohydrate source. Two other cellulolytic ruminal bacteria, *Ruminococcus flavefaciens* and *Fibrobacter succinogenes*, are also xylanolytic, but some strains of these species do not use xylose per se for growth (1, 13). Like these other cellulolytic bacteria, xylan degradation by *R. albus* facilitates its access to and use of plant celluloses as a carbohydrate source, and at least some of the xylan degradation products are used by other ruminal bacteria. Cross-feeding between ruminal bacteria has long been recognized (4), and some strains of the nonxylanolytic bacterium *Selenomonas ruminantium* use xylooligosaccharides produced by xylanolytic bacteria such as *Butyrivibrio fibrisolvens* (6, 7). However, given that cellulose degradation by *R. albus* is maximal in the presence of PAA/PPA, the preservation of this conditional expression of cellulase activity suggests that an additional symbiotic relationship(s) underpins the role of *R. albus* in ruminal polysaccharide degradation.

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